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# Flow cytometric method for the routine follow-up of red cell populations after bone marrow transplantation

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**Summary.** A simple and sensitive flow cytometric method is presented for the quantitation of erythrocyte subpopulations. Cells were indirectly labelled using human antisera (mostly crude) and fluorescein isothiocyanate (FITC)-conjugated anti-human IgG F<sub>ab</sub>. The method was evaluated by analysing artificial mixtures of blood group antigens A, B, D, E, c, K and Fy<sup>a</sup>. Intra-assay coefficients of variation were found to be 7.2% for 1% D-positive mixtures and 2.2% for

10% D-positive mixtures; the inter-assay coefficients of variation were 9.5% and 6.0% respectively. The sensitivity of the method was found to be 0.31%. The method has shown to be suitable for the routine follow-up of patients after allogeneic bone marrow transplantation (BMT).

**Keywords:** flow cytometry, bone marrow transplantation, red blood cells.

The quantitation of autologous and donor erythrocyte subpopulations in patients after allogeneic BMT was determined by following the repopulation of red cells, identification of relapses at an early stage, and the detection of long-term chimaerism. We have previously described a method to detect erythrocyte chimaerism using fluorescent microspheres (de Man *et al*, 1988) with a sensitivity of 0.01% positive cells.

Flow cytometry (FC) is an analysis method increasingly used for the detection of erythrocyte subpopulations (Bayliss *et al*, 1991; Blanchard *et al*, 1995). The flow cytometer analyses large numbers of cells within a short time. Therefore it is especially useful for detecting minor cell populations. For more objective and reproducible results we investigated the use of FC analysis for the quantification of erythrocyte subpopulations. Blanchard *et al* (1995) have described a method for this purpose using blood group antigens A, B and H as population markers.

We present a method suitable for a wide range of blood group antigens as erythrocyte population markers. Results are shown for the markers A, B, D, E, c, K and Fy<sup>a</sup>. Using human antibodies (mostly crude sera) in a simple two-step procedure with FITC-conjugated F<sub>ab</sub>, our method is much easier than the one described by Blanchard *et al* (1995)

which requires an extensive preparation of monoclonal, FITC-conjugated antibodies. Furthermore, fixation of the erythrocytes is rendered unnecessary by the use of IgG antibodies in combination with anti-human IgG F<sub>ab</sub>.

## MATERIALS AND METHODS

**Antisera.** Crude human sera containing IgG antibodies anti-E, -c, -K and -Fy<sup>a</sup> were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam. Anti-K was diluted 1:8 with phosphate-buffered saline containing 0.1% w/w bovine serum albumin (Sigma, St Louis, U.S.A.) (PBS-BSA). The other sera were used undiluted. Anti-D recommended for the bromelin test, was obtained from the same institute and was used in a 1:16 dilution.

The IgG fractions of anti-A and anti-B were purified from high titre sera of patients on a Protein G Sepharose 4 fast flow (Pharmacia) affinity column according to the recommendations of the manufacturer. The concentrates were used at a dilution of 1:12 and 1:4 respectively.

**Erythrocytes.** Erythrocyte subpopulations were quantified in artificial mixtures of antigen positive and negative red blood cells (RBC) as well as in blood samples of patients after BMT. The subpopulations were distinguished using the blood group antigens A, B, D, E, c, K or Fy<sup>a</sup>. Blood of healthy volunteers was used for the artificial mixtures. The positive RBC were chosen to be heterozygous for the marker antigen

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(except antigen A and B). The phenotype R<sub>1</sub>r was used for D-antigen positive cells.

Fresh and stored blood samples of patients were analysed. Patients with leukaemia were transplanted with T-cell-depleted bone marrow as described by Schattenberg *et al* (1990). Blood was drawn before and several times after BMT.

Before labelling, most of the leucocytes were removed by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). For this purpose, one volume of blood was mixed with one volume of PBS-BSA. This mixture was centrifuged over 2 ml Ficoll-Paque (1.077 g/ml), and the RBC pellet was washed three times.

Most of the leucocytes were removed before sample storage. The erythrocytes were resuspended in 30% glycerol with 159 mM sorbitol and 108 mM NaCl and kept frozen in liquid nitrogen. After thawing, the samples were cleared of lysed cells by washing and centrifugation over Ficoll-Paque before labelling.

**Labelling of erythrocytes.** RBC were labelled with human IgG antibodies directed against the corresponding antigen by adding 50 µl of the suitable antiserum to 200 µl of an RBC suspension with a concentration of  $1.25 \times 10^8$  cells/ml. After 1 h incubation at 37°C, the RBC were washed three times with 4 ml PBS-BSA. In round-bottomed 96-well microtitre plates  $7 \times 10^5$  cells were pelleted, resuspended in 70 µl 1:128 diluted FITC-conjugated anti-human IgG-F<sub>ab</sub>

(Cappel, Durham, U.S.A.), and incubated at room temperature for 30 min. After washing three times with 200 µl PBS-BSA, the cells were resuspended in 1 ml PBS-BSA and analysed with the flow cytometer.

Red cells were briefly fixed prior to labelling with anti-A. This was achieved by incubation with glutaraldehyde (0.1% in PBS) for 10 min.

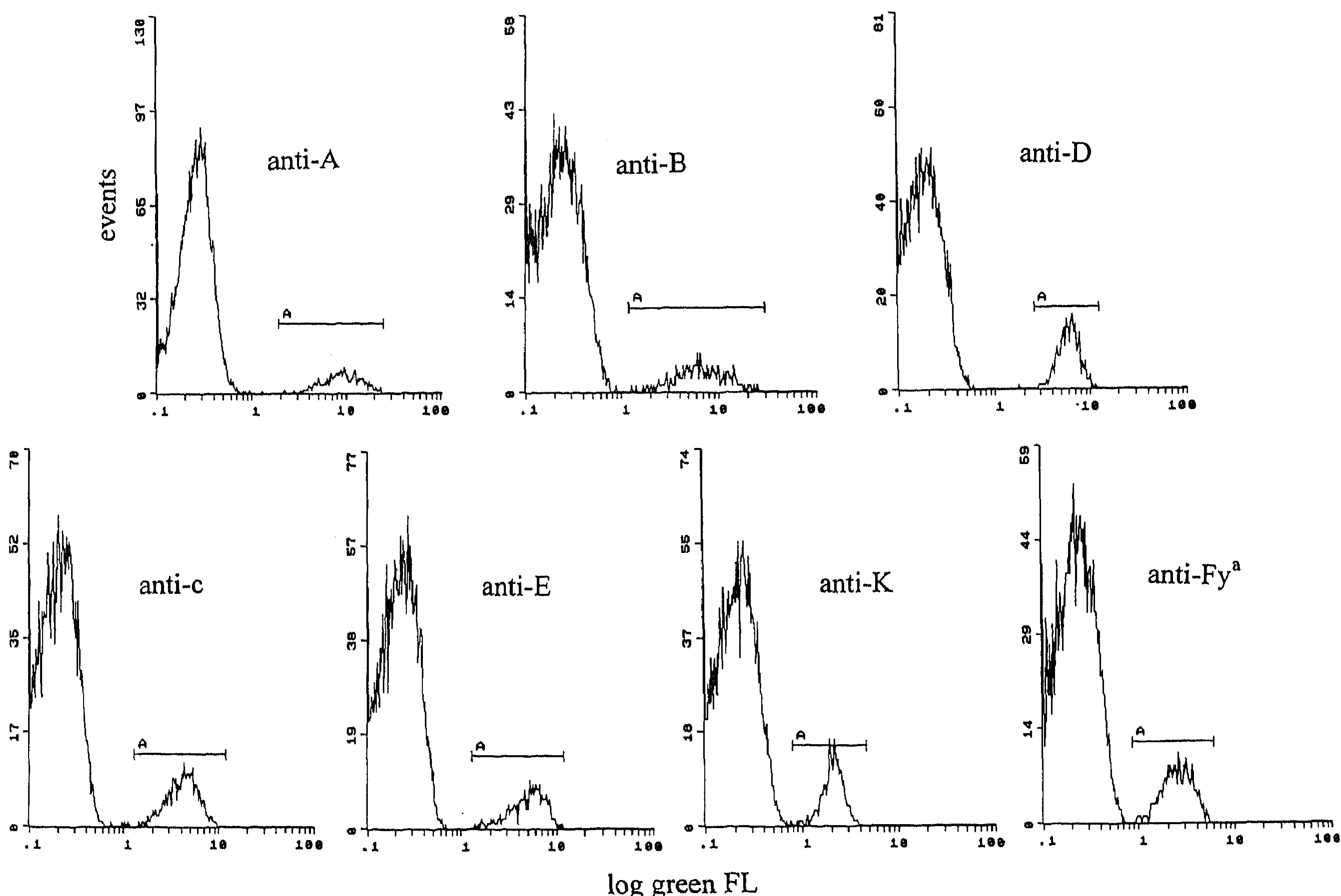
**Flow cytometry.** Analysis was performed on a Epics-XL flow cytometer (Coulter Electronics) equipped with a 15 mW argon laser and standard filter setting. Fluorescent signals were logarithmically amplified. Data were handled with Elite Software (Coulter Electronics). RBC were identified and selected according to their forward and side scatter profiles. For samples with low percentage positive cells, the positive region is set with the analysis of a 1% positive artificial mixture of the corresponding antigen.

Depending on the percentage cells found to be positive for the marker antigen, the following number of cells were analysed: 25 000 (<5% positive cells) or 10 000 cells (>5%) at a flow rate of 250–350 cells/s.

## RESULTS

### Artificial mixtures

Mixtures of antigen positive and negative RBC were prepared at different concentrations and analysed by the new



**Fig 1.** Fluorescence intensity histograms of artificial mixtures of 10% antigen-positive and 90% antigen-negative erythrocytes. A-positive cells had the A<sub>1</sub> phenotype; D-positive RBC had the R<sub>1</sub>r phenotype, E-, c-, K- and Fy<sup>a</sup>-positive RBC used were heterozygous for the antigen.



**Table I.** Comparison of determined percentages of antigen-positive cells to expected percentages in artificial mixtures of antigen positive and negative red cells. D-positive RBC had the R<sub>1</sub>r phenotype. A-positive cells had the A<sub>2</sub> phenotype. E-, c-, K- and Fy<sup>a</sup>-positive RBC used were heterozygous for the antigen.

Expected percentage	Determined percentage						
	D	E	c	K	Fy <sup>a</sup>	A	B
0	0.05	0.11	0.12	0.14	0.09	0.10	0.05
0.1	0.12	0.16	0.12	0.21	0.13	0.17	0.14
0.3	0.37	0.41	0.42	0.44	0.29	0.33	0.26
1.0	0.97	0.96	1.1	0.96	1.2	0.92	0.73
3.0	3.1	2.8	3.1	2.9	2.9	2.6	1.74
10	9.4	8.5	10	9.0	9.5	8.5	7.84
30	29	28	29	25	28	26	30
75	n.d.	70	75	76	n.d.	65	74
100	100	100	100	100	98	n.d.	99

n.d. = not determined.

technique. The fluorescence intensity of the positive cells depended on the antigen but was always clearly distinct from that of the negative cells (Fig 1).

Agglutination occurred during centrifugation after sensitization with anti-A antibodies. This resulted in an incorrect analysis. The problem was solved by the introduction of a brief fixation stage prior to labelling with anti-A.

The blank of the method was  $0.09 \pm 0.03\%$ . Unlike the 0.1% positive mixtures (mean 0.15%), the 0.3% positive mixtures (mean 0.36%) were clearly distinct from the blank (Table I). The determined percentages of positive RBC were in

good agreement with the expected percentages of the artificial mixtures.

The reproducibility was determined by replicate analysis of artificial mixtures of 1% and 10% D-positive mixtures. The intra-assay results were  $1.08 \pm 0.08$  (SD)% ( $n = 10$ ) and  $9.4 \pm 0.2$  (SD)% ( $n = 10$ ) with coefficients of variation of 7.2% and 2.2% respectively. The inter-assay results were  $1.03 \pm 0.10$  (SD)% ( $n = 10$ ) and  $9.2 \pm 0.6$  (SD)% ( $n = 6$ ) with coefficients of variation of 9.5% and 6.0% respectively.

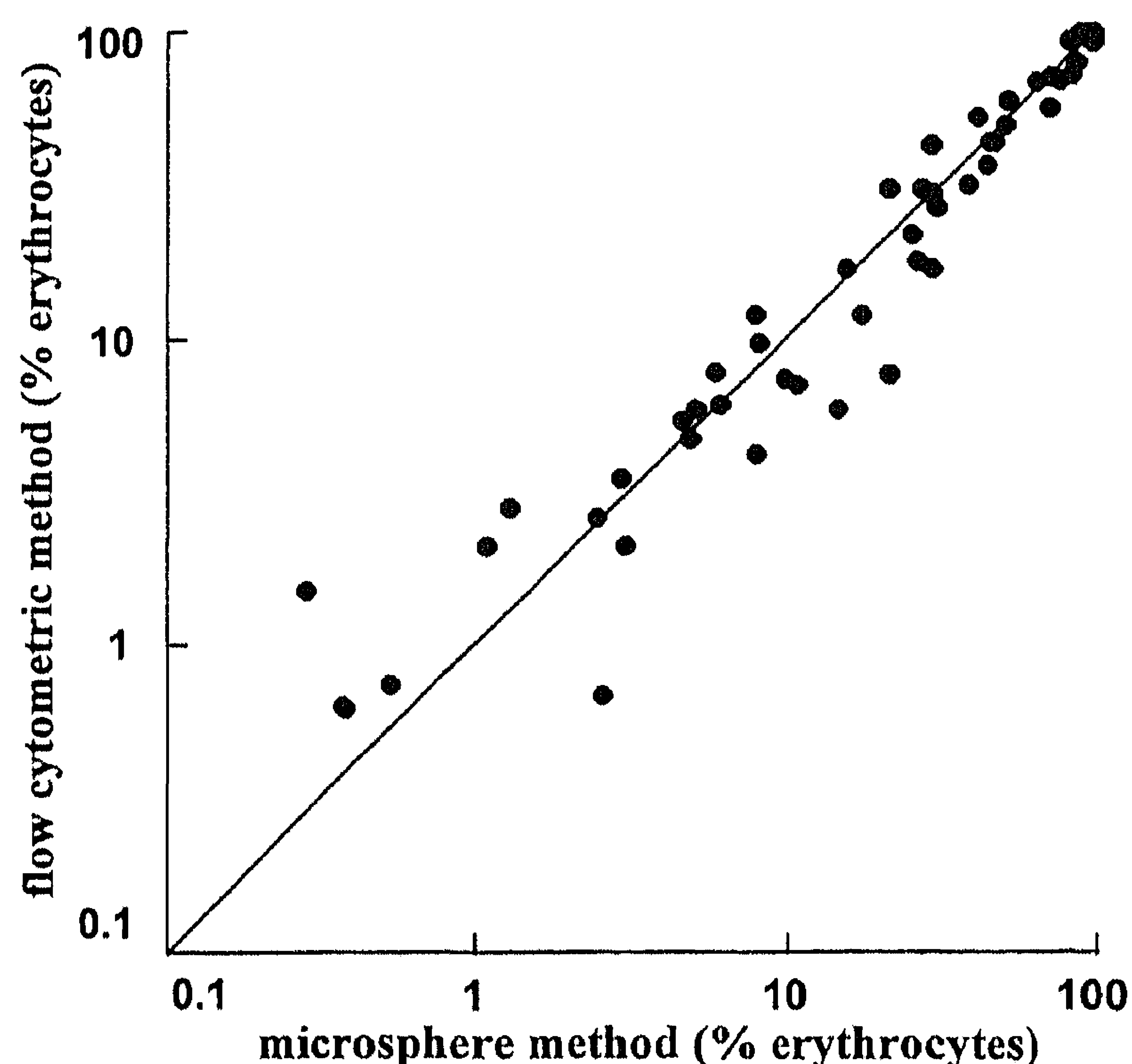
#### Patient samples

The sensitivity of the method was tested by assaying 14 samples drawn from patients just before BMT. All stated antigens except B were used as markers. The percentage false positive cells in these samples ranged from 0.04% to 0.25% with a mean of  $0.12 \pm 0.06$  (SD)%. The detection limit deduced from this result was 0.31% (mean + 3 SD).

A total of 54 positive blood samples of nine patients after BMT were analysed by the new technique as well as with the microsphere method described by de Man *et al* (1988). The correlation between both methods, shown in Fig 2, was excellent ( $r = 0.96$ ).

The new flow cytometric method was used to monitor the percentage of donor and autologous erythrocytes after allogeneic BMT in patient blood samples. The data of eight representative patients with a successful BMT are shown in Fig 3. We monitored the donor erythrocytes of five patients and the autologous erythrocytes of four patients. In one patient both the donor erythrocytes (antigen K) and autologous erythrocytes (antigen D) were followed.

Regarding donor erythrocytes, there always was a clear increase in the percentages at 14 d after BMT compared to the percentages on the day of transplantation. In those cases where the autologous erythrocytes were followed, the percentages declined in the period up to 120 d after BMT. This is concordant with a successful destruction of



**Fig 2.** Correlation of the percentages of antigen-positive erythrocytes in blood samples of patients after BMT obtained by the presented method and by microsphere method. Antigens used for labelling were D, E, c, K and Fy<sup>a</sup>. Line of correlation found is  $y = 1.03x - 0.02$ , with a correlation coefficient of 0.96 ( $n = 54$ ).



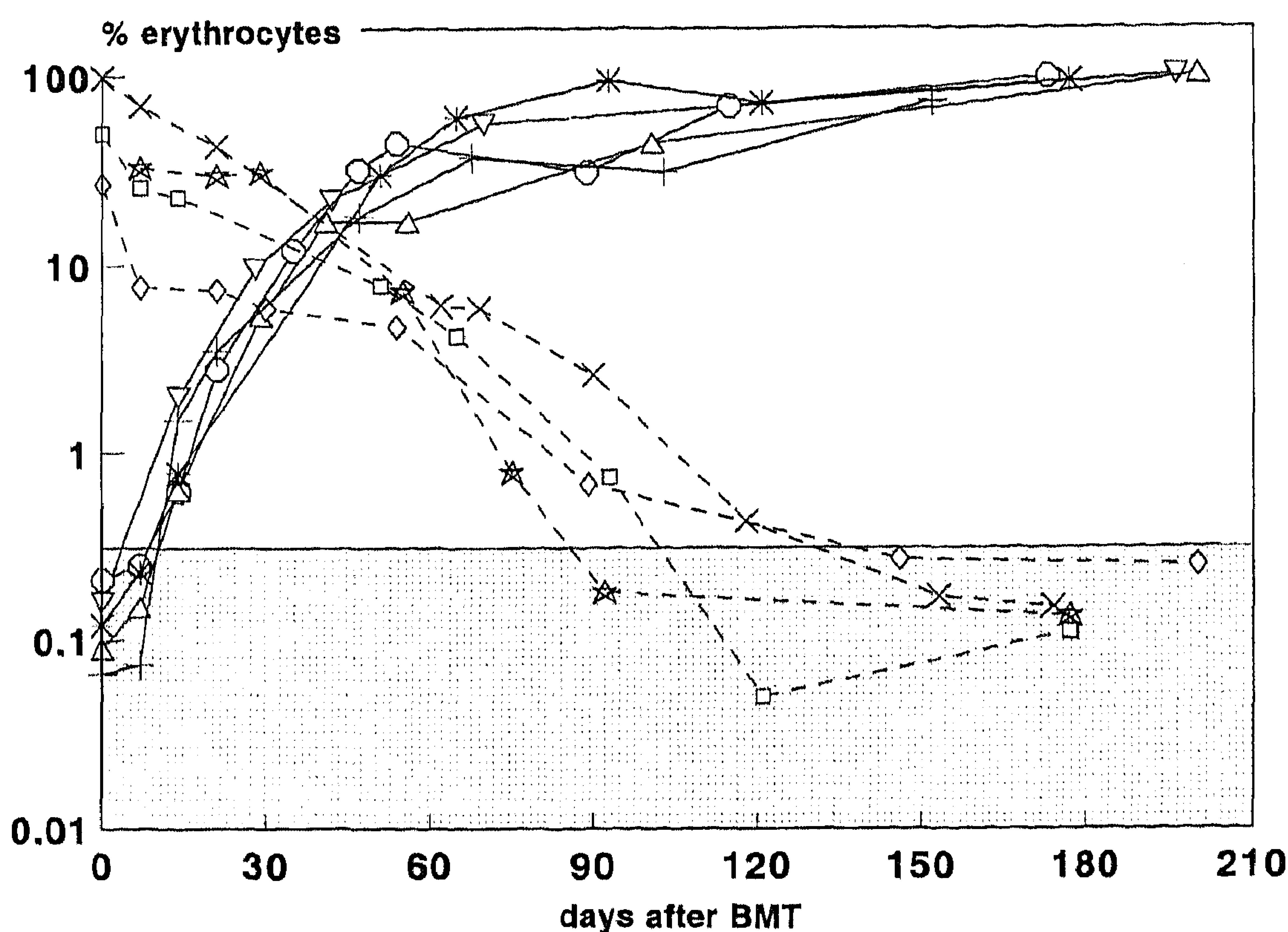


Fig 3. Percentages of donor (—) and autologous (---) erythrocytes determined in blood samples of patients at various time intervals after BMT. Antigens used as markers were D (+ and □), E (◇), c (○ and ×), K (☆, ∇ and \*) and Fya (Δ). In one patient, the donor (\*) as well as the autologous (□) erythrocytes were followed. Values in the shaded area are below the detection limit of 0.31%.

the autologous bone marrow and a 120 d survival of erythrocytes in peripheral blood.

## DISCUSSION

The new flow cytometric method used blood group antigens A, B, D, E, c, K and Fy<sup>a</sup> to quantify erythrocyte subpopulations. The antigen of choice was indirectly labelled with FITC. We chose indirect labelling to avoid the conjugation of the antibodies with FITC. Another advantage of indirect labelling was the amplification of the signal (Garratty & Arndt, 1995).

To avoid agglutination occurring in the second step of the labelling procedure, we used F<sub>ab</sub> of anti-human IgG instead of the whole molecule. We preferred this strategy over the time-consuming overnight fixation described by Blanchard *et al* (1995). Agglutination occurred after the first labelling step with anti-A antibodies, although only IgG antibodies were used. This agglutination was prevented by a mild and brief (10 min) fixation with glutaraldehyde.

Fluorescence intensity of the positive RBC differed among the antigens used as marker. This was mainly caused by differences of antigen density between blood group antigens. The affinity for the antigen and the concentration of the antibodies also played a role. The detection limit of 0.31% was deduced from the results with patient blood samples drawn just before BMT ( $t = 0$  d). This result was confirmed by the percentages of autologous erythrocytes found in samples drawn 120 d after BMT or later. All these values were below the detection limit. The mean of the negative patient samples ( $0.12 \pm 0.06\%$ ) was higher than the blanks with blood of healthy volunteers (mean  $0.09 \pm 0.03\%$ ). This effect was probably caused by the medication and/or the disease of the patients.

Most of the described flow cytometric methods for analysis of erythrocyte subpopulations are used to determine the

extent of feto-maternal haemorrhage (FMH) and use solely antigen D as marker (Nance *et al*, 1989; Patton *et al*, 1990; Bayliss *et al*, 1991). Sensitivities of 0.1–0.2% have been achieved. Blanchard *et al* (1995) developed a flow cytometric method to detect erythrocyte subpopulations after BMT. They label antigen A, B and H directly with FITC, thereby reaching a sensitivity level of 1%.

Our method is simple, accurate and sensitive. A series of 10 samples can be processed within 5 h. Furthermore, little time is needed for the preparation of the reagents. The method has been shown to be appropriate for the routine follow-up of patients after BMT with each of the presented seven markers. All five examples following donor erythrocytes had a detectable percentage erythrocytes in the samples drawn 14 d after BMT. The earliest detection of donor erythrocytes that Blanchard *et al* (1995) report is 24 d after BMT. It is unclear whether or not they evaluated samples drawn 14 d after BMT.

A precondition for application of this flow cytometric method, which is based on blood group differences, is the transfusion policy that only RBC negative for the marker antigens are transfused. This policy must be applied at least 120 d before sampling.

To be able to investigate more patients, the number of suitable marker antigens has to be extended (C, Fy<sup>b</sup>, Jk<sup>a</sup>, Jk<sup>b</sup>, M, N, S and s). With the expanded set of marker antigens, almost all patients after BMT can be monitored. Other potential applications of the presented method are detection of FMH, survival studies of transfused erythrocytes, and studies of natural occurring erythrocyte chimaerism and mosaicism.

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